New perspectives on bioactivity of olive oil – evidence from animal models, human interventions and the use of urinary proteomic biomarkers

By S. Silva[1,2,3], E. Combet[4], M.E. Figueira[3,5], T. Koeck[6], W. Mullen[7] and M.R. Bronze[1,2,3,5],

[1]iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal [2] ITQB, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal [3]Pharmacy Faculty, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal, [4]Human Nutrition, School of Medicine, University of Glasgow, Glasgow G31 2ER, UK [4]iMED.Ulisboa, Research Institute for Medicines, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal [6] Mosaiques diagnostics GmbH, Mellendorfer Strabe 7-9, 30625, Hannover, Germany, and [7]Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow G12 8QQ, UK

This is a post peer review manuscript of the full paper published in *Proceedings of the Nutrition* 08/2015; 74(3):268-281. DOI:10.1017/S0029665115002323

Corresponding author:

M. R. Bronze, Pharmacy Faculty, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal; telephone number (+351) 21 794 64 00 Ext. 14329; Fax number (+351) 217946470

email mrbronze@ff.ulisboa.pt.

Short title: New perspectives on bioactivity of olive oil

Keywords: olive oil, phenolics, coronary artery disease, inflammation, proteomic biomarkers

Abstract

Olive oil (OO) is the primary source of fat in the Mediterranean diet and has been associated with longevity and a lower incidence of chronic diseases, particularly coronary heart disease. Cardioprotective effects of OO consumption have been widely related with improved lipoprotein profile, endothelial function and inflammation, linked to health claims of oleic acid and phenolic content of OO. With cardiovascular disease being a leading cause of death worldwide, a review of the potential mechanisms underpinning the impact of OO in the prevention of disease is warranted. The current body of evidence relies on mechanistic studies involving animal and cell-based models, epidemiological studies of OO intake and risk factor, small and large scale human interventions, and the emerging use of novel biomarker techniques associated with disease risk. While model systems are important for mechanistic research nutrition, methodologies and experimental designs with strong translational value are still lacking. This review critically appraises the available evidence to date, with particular focus on emerging novel biomarkers for disease risk assessment. New perspectives on OO research are outlined, especially those with scope to clarify key mechanisms by which OO consumption exerts health benefits. The use of urinary proteomic biomarkers, as highly specific disease biomarkers, is highlited towards a higher translational approach involving olive oil in nutritional recommendations.

1. Relevance of the Mediterranean diet and olive oil to health

The olive tree, *Olea europaea* L., is one of the oldest agricultural tree crops and provides diversified products for human consumption such as table olives and olive oil (OO)[1]. The analytical parameters to ascertain OO quality and classify OOs are defined by European Union (EU) regulations[2]. Oils obtained only by mechanical extraction are virgin olive oils (VOOs) and further quality assessment can lead to a classification as extra virgin olive oil (EVOO)[3].

OO is the primary source of fat in the Mediterranean diet and has been associated with longevity and a lower incidence of chronic diseases, particularly coronary heart disease (CHD)[4-7]. OO consumption is also associated with decreased rates of cancer, diabetes and neurodegenerative diseases[8] as well as body weight reduction and obesity prevention[9, 10]. The epidemiological evidence underpinning the relevance of the Mediterranean diet to health is strong with over seventeen studies including 2300 volunteers confirming that a Mediterranean diet decreases inflammation and improves endothelial function[11], and a meta-analysis of thirty-two cohort studies (> 800,000 subjects) indicating that there is an inverse correlation between OO intake and coronary heart disease[12].

Olive oil bioactive components

The major components of OO are glycerols (saponifiable fraction) which represent more than 98% of of the total oil weight and are mainly triglyceride esters of oleic acid (55 to 83%), palmitic acid (7.5 to 20%), linoleic acid (3.5 to 21%) and other fatty acids such as stearic acid (0.5 to 5%)[13]. Minor components (the unsaponifiable fraction) include aliphatic and triterpenic alcohols, sterols, hydrocarbons as squalene, volatile compounds, tocopherols, carotenes, chlorophyll and phenolic compounds[13-15].

Special attention has been given to the phenolic compounds only found in VOO and EVOO. The agronomic and technological aspects of OO production have an impact on the concentration of phenolic compounds, as does the pedoclimatic conditions and agronomic techniques (e.g.: irrigation)[4, 14]. The main classes of phenolic compounds present in VOO are phenolic acids, phenolic alcohols (hydroxytyrosol and tyrosol), flavonoids, lignans and secoiridoids, **Table 1**.

Oleuropein and ligstroside, the most significant secoiridoids in *Olea europaea* L., are esters of elenolic acid glucoside with hydroxytyrosol and tyrosol, respectively. During the mechanical

extraction of the oil, fruit endogenous β-glucosidases[14, 16] are released leading to the secoiridoid aglycones formation, accounting for more than 50% of the phenolic content of the oil[17, 18]. The most abundant secoiridoids of VOO are the oleuropein and ligstroside aglycons and dialdehydic forms of deacetoxy of oleuropein and ligstroside aglycons[14] also named oleacein and oleocanthal, respectively[19].

Phenolic compounds bioavailability and bioactivity

Once OO has been ingested, it produces a micellar solution composed of a lipid and an aqueous phase. Chemical hydrolysis of secoiridoids can take place in the acidic medium of the stomach[20] or in alkaline conditions in the small intestine[21, 22] leading to an increase of free phenolic alcohols released into the aqueous phase. As a result OO phenolic compounds are further absorbed in the small intestine[23]. Measuring the bioavailability of these compounds in plasma and urine reveals that OO phenolics undergo a conjugation process of methylation, glucuronidation and sulfation indicating that there is phase 2 metabolism involved during the absorption of these compounds[24-27]. The between-subjects variability in human absorption and metabolism of OO phenolics may explain differences in proportion of methyl, glucuronide and sulfate conjugates reported[28-30].

Bioavailability of OO phenolic compounds differs according to the intake matrix. OO as the intake vehicle promotes absorption of hydroxytyrosol: the corresponding bioavailability of hydroxytyrosol in rats for aqueous and OO solutions were reported as 75 and 99%[31], respectively. When a supplement containing hydroxytyrosol as a single oral dose (2.5 mg/kg) was fed to humans, the bioavailability was below 10%[32], while previous studies showed higher bioavailability for hydroxytyrosol supplementation in lipid vehicles[33]. The addition of hydroxytyrosol to low fat yogurt and administered to humans was also associated with a lower excretion of hydroxytyrosol when compared with OO[33]. As OO phenolic compounds are mainly absorbed in the small intestine[23] the increase of hydroxytyrosol bioavailability, in OO, might be related to the rate of gastric emptying[32] and slow release of hydroxytyrosol from the oil matrix[26, 32]. The presence of other antioxidants in OO might prevent breakdown of hydroxytyrosol before absorption in the gastrointestinal tract[31].

Secoiridoids that are not absorbed in the small intestine are degraded by the colonic microbiota with oleuropein producing hydroxytyrosol as the major product[20]. *In vitro* colonic metabolism was evaluated on tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein showing an increase in phenolic acids, stability of hydroxytyrosol and tyrosol and degradation of hydroxytyrosol acetate and oleuropein mainly to hydroxytyrosol[34]. In order to evaluate OO phenolic metabolites produced from colonic fermentation, faecal samples were analysed before and after mid-term consumption of phenol-rich OO[34]. A significant increase in hydroxytyrosol concentration ($p < 0.05$) was observed after phenol-rich OO intake. Although absorption of OO phenolic compounds mainly occurr in the small intestine a small proportion of hydroxytyrosol and its derivatives still pass into the large intestine[23]. This highlights the need to study the impact of OO phenolics in the colon, either with gut microbiota interaction or local activity due to its antioxidant and anti-inflammatory properties.

When assessing the chemical and *in vitro* biological antioxidant activities of these compounds, it is the glucuronides conjugates of hydroxytyrosol and tyrosol that must be assessed. These were tested in the range 0.01–10 μM against the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). None of the glucuronides displayed significant antioxidant activities at the concentrations tested, whereas the parent aglycones did display antioxidant activity at these concentrations[35]. This conflicts with the results of others[36] with differences attributed to the fact that in one study reference standard material[35] was used and in the other the glucuronide conjugates were extracted from urine samples[36], and likely contained inpurities that had antioxidant activity. Hydroxytyrosol metabolites might act as "sinks" of hydroxytyrosol that could be locally released in the cells after enzymatic hydrolysis[37], therby explaining the proposed hydroxytyrosol biological effects observed *in vivo*. Moreover, *in situ* deconjugation of hydroxytyrosol metabolites (into their free form) in red blood cells was observed in rats after oral administration of an OO phenolic extract obtained from olive cake (1.5 g/kg body weight, equivalent to 34.4 mg of hydroxytyrosol and derivatives), highlighting a potential protective mechanism against cell oxidative damage[38].

Although there are a number of biological effects for OO phenolic compounds, most cannot be achieved via normal dietary exposure to OO. This has led to development of enriched products with natural OO phenolic compounds. OO by-products such as olive mill wastewater[39] and

olive pomace[40, 41] are potential sources of natural bioactives which could be used to supplement OO. The development of new OO products such as pomace OO or refined olive oil (ROO) enriched in natural bioactives opens new perspectives in the field.

2. Olive oil and inflammation

Inflammation involves a complex cascade of events partly related with the production of an excess of free radicals due to internal or environmental stress[42]. The inflammation process triggers signaling molecules such as nuclear factor-kappa-B (NF-$_k$B), which up-regulates the production of inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α)[43] inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and interleukin-1beta (IL-1β)[42].

A number of phenolic compounds present in OO have anti- inflammatory properties, including oleocanthal, a secoiridoid (dose-dependent inhibition of COX-1 and COX-2 activities, similar to the anti-inflammatory drug ibuprofen[44]). However, to achieve comparable effect to the recommended daily dose of ibuprofen, 500 g of EVOO would need to be consumed[45, 46] making the dose/effect relationship outwith any (acute) inflammatory benefits due to typical OO consumption.

Chronic inflammation

Rheumatoid arthritis (RA) is a major inflammatory, autoimmune, disease characterized by chronic joint inflammation[47, 48]. Hydroxytyrosol has been studied for its anti-inflammatory effects in a RA animal model. We reported that it provided beneficial effects in the evolution of the disease[49], with 0.5 and 5 mg/kg doses in rats, after gavage administration, using ROO as vehicle (human-equivalent of 4.9 and 49 mg/day, respectively, for a 60 kg adult), **Figure 1**. Significant effects, on paw edema reduction, were observed for a human-equivalent dose of 49 mg/day, a dose 10 times higher than the approved European Food Safety Authority (EFSA) dose for phenolic compounds in relation to protection of lipid oxidation[50]. The same hydroxytyrosol dose was effective on colitis, another chronic inflammatory disease[51]. This dose would only be achievable through nutraceutical supplementation of OO with hydroxytyrosol, and the use of this functional food on a daily basis.

To further evaluate the anti-inflammatory mechanisms involved with hydroxytyrosol, we studied COX-2 and iNOS expression[49]. The treatment at 5 mg/kg dose significantly decreased histological damage, COX-2 and iNOS expression (p<0.001 *vs.* positive control), markedly

reduced the degree of bone resorption, soft tissue swelling and osteophyte formation, improving articular function in treated animals. Moreover at the same dose there was a significant decrease (p<0.005 *vs.* positive control and ROO) in TNF-α serum levels. These results are in line with others that reported benefits on RA, in animal models, after oral administration of an EVOO extract[52], intraperitoneal administration of oleuropein aglycone[53] or polyphenol supplemented VOOs diets[54]. The reports highlight effects on RA of OO phenolic compounds either administered as isolated compounds or as an extract. However, doses comparison between animal studies have to to take in consideration not only differences in species (rats *vs.* mice) but also routes of administration. Compared to intraperitoneal administration, an oral dose has an extra pass through the liver with consequent metabolism through the first-pass effect.

Acute inflammation

Acute inflammation has been commonly induced using carrageenan in animals in order to evaluate the effects of non steroid anti-inflammatory drugs (NSAIDs)[55]. We studied the effect of hydroxytyrosol-supplemented OO on acute inflammation, induced by carrageenan in rats, at 0.5 and 5 mg/kg[49] dose, after gavage administration which occurred 30 min before the challenge with carrageenan. Both doses significantly reduced paw edema (p<0.001 *vs.* positive control) with the lowest effective dose being achievable through OO daily intake. Previous studies in rats[56] also showed inhibition of carrageenan - acute inflammation of an aqueous hydroxytyrosol formulation (HT-20, 22% hydroxytyrosol), and significant effects were obtained at a 22 mg/kg hydroxytyrosol dose. Differences in dose effect might be related to the administration vehicle with ROO or OO being better vehicles than water.

3. Cardioprotection of olive oil

Most of the interventional studies focusing on the benefit of VOO intake on cardiovascular disease have investigated the effect of phenolic compounds on the prevention of oxidation of low-density (LDL) and high-density (HDL) lipoproteins[57-64], two risk markers of cardiovascular disease. A number of trials have also focused on cardioprotection against inflammation[65] mainly on antioxidant activity and inflammatory mediators.

Impact of olive oil constituents on lipoproteins and atherosclerosis

Fat content

LDL particles carry about two-thirds of plasma cholesterol and can infiltrate the arterial wall attracting macrophages, smooth muscle cells, and endothelial cells[66] thus driving atherosclerosis. LDL particle size is influenced by type and amount of dietary fat consumed[67]: Low-fat diets lead to a decrease in the size of LDL particles compared to high-fat diets[68]. The type of fat ingested is also important: LDL particles are larger with high-monounsaturated fatty acid diets (such as those based on OO), compared to diets with a high polyunsaturated fatty acids intake, where LDL particles are smaller[69]. LDL particle size is especially relevant, since small size particle are more prone to oxidation and can better enter into the arterial wall when compared with larger LDL particles[70]. Conversely, HDL particles are antiatherogenic, as their primarily role is to deliver cholesterol to the liver to be metabolized and excreted or reused. HDL may also be able to dislodge cholesterol molecules from atheromas in arterial walls[66]. It has been reported in patients with peripheral vascular disease[71, 72], that LDL particles are less susceptible to oxidation when the diet is enriched in VOO monounsaturated fatty acids, compared to the polyunsaturated fatty acids of sunflower oil enriched diets. Moreover when compared to saturated fatty acids intake, OO oleic acid reduces the level of LDL-cholesterol[63, 64].

The health benefits associated with monounsaturated fat content in OO were recognised by the United States Food and Drug Administration (FDA) in 2004, highligthing "the benefits on the risk of coronary heart disease of eating about two tablespoons (23 g) of OO daily"[73]. Health benefits were related with a decrease of total and LDL cholesterol in serum[73], diet improvement of endothelial dysfunction[74], coagulation activity[75] and reduced LDL susceptibility to oxidation[72].

Phenolic content

Antioxidants that can prevent lipid peroxidation, such as phenolic compounds, could play an important role in preventing oxidative modification of LDL[4], with the oxidative process an initiating factor for atherosclerotic plaques[76]. Once monocytes differentiate in macrophages on the endothelium they scavenge oxidized LDL (ox-LDL), then becoming foam cells, leading to plaque formation[5].

The Effect of Olive Oil on Oxidative Damage in European Populations (EUROLIVE) study was a cross-over fat replacement intervention[58], using OOs with different phenolic content in healthy male volunteers. Its findings led to the current EFSA recommendation (Opinion of the Scientific Committee/Scientific Panel, *EFSA Journal*[50, 77, 78]). A linear increase in HDL-cholesterol levels after 3 weeks was observed after low-, medium-, and high-polyphenol OO consumption: mean change from preintervention, 0.02 mmol/L (95% CI, 0.00 to 0.05 mmol/L), 0.03 mmol/L (95% CI, 0.00 to 0.05 mmol/L) , and 0.04 mmol/L (95% CI, 0.02 to 0.06 mmol/L), respectively. Total cholesterol:HDL cholesterol ratio decreased linearly with the phenolic content of the OO. Triglyceride levels decreased by an average of 0.05 mmol/L for all OOs[58]. Mean changes from preintervention for ox-LDL levels were 1.21 U/L (95% CI, -0.8 to 3.6 U/L), -1.48 U/L(95% CI, -3.6 to 0.6 U/L) and -3.21 U/L (95% CI, -5.1 to -0.8 U/L) for the low-, medium-, and high-polyphenol OO, respectively, showing a dose-dependent relation with VOO phenolic content[58]. The EFSA confirmed a cause effect relationship between consumption of OO phenolics (standardized by the content of hydroxytyrosol and its derivatives) and protection of LDL cholesterol particles against oxidative damage. To support the EFSA health claim, 5 mg of hydroxytyrosol and its derivatives should be consumed daily in 20 g OO[50], but concentrations in some OOs may be too low to achieve this target in the context of a balanced diet. Moreover, the EFSA Panel commented study design limitations as most human interventions with OO have been conducted in more homogeneous male populations[77] and not in general population.

The contribution of OO phenolics toward cardiovascular health benefits has been challenged with inconsistent results reported for *ex vivo* resistance of LDL to oxidation[79, 80]. Seven human intervention studies with OO were compared for impact of phenolics on ox-LDL, with no effect seen in five of them[79], possibly explained by artifacts generated during LDL isolation.

Since the approval of the EFSA claim, both terminology and analytical methodology supporting the dose calculation of hydroxytyrosol and derivatives have been appraised. Mastralexi *et al.*[81] commented on the weaknesses of the claim terminology namely the term “olive oil polyphenols” is not entirely clear and accurate as “olive oil” is a generic term for the type of oil, and the basic structure of OO phenolic compounds do not coincide with a “polyphenolic” structure; accordingly “virgin olive oil bioactive phenols” is a more appropriate term. Others also

commented about the lack of robust and reliable methods for quantifying phenolic compounds in OO. A simple and robust method for routine analysis of hydroxytyrosol and tyrosol was proposed[81, 82] based on hydrolysis of the polar fraction of OO. This was followed by development and validation of a ^{1}H NMR method enabling direct measurement of tyrosol and hydroxytyrosol derivatives, as well as oleocanthal and oleacein in OO, overcoming analytical issues such as chromatographic peak broadening[19].

Cardioprotective mechanisms of oleic acid

OO intake has been related with a decrease on blood pressure with oleic acid regarded as being a major contributor to this effect, as evidenced in animal models[83]. Chronic oral administration of VOO (rich in oleic acid), triolein (a triacylglyceride with three oleic acid moieties) or oleic acid over 14 days significantly reduced systolic blood pressure in rats (-26 ± 4 for VOO and -21 ± 3 mm Hg for triolein, $p<0.001$, and -17 ± 1.9 mm Hg for oleic acid $p<0.05$) when compared to the control group that received water. Similarly acute (2 h) treatments with either VOO or triolein also significantly reduced systolic blood pressure when compared to the control group (-20 ± 0 mm Hg, $p < 0.001$, and -14 ± 2 mm Hg, respectively, $p < 0.05$) with oleic acid again significantly reducing systolic blood pressure (-13.0 ± 0.3 mm Hg; $p < 0.001$). In contrast, chronic treatment with the trans-monounsaturated fatty acid elaidic (18:1n-9) or the saturated fatty acid stearic acid (18:0) did not significantly affect blood pressure. Results show that saturation and cis/trans double bond arrangement are implicated with the cardioprotective effect of the long chain fatty acid in this animal model at high dose levels[83]. Similar significant results were obtained after VOO and oleic acid intake in an animal model of hypertension using spontaneously hypertensive rats[83].

The molecular mechanisms were evaluated by measuring signaling proteins involved in the control of blood pressure in the aorta. OO intake increases oleic acid levels in membranes, which regulate membrane lipid structure and impact on G protein-mediated signaling, causing a reduction in blood pressure[84]. Unlike its analogues elaidic and stearic acid, oleic acid, due to its cis-18:1n-9 structure, regulates cellular membrane lipid structure and the α_2 receptor system involved in the control of blood pressure ($\alpha_{2A/D}$ - adrenoreceptor/G protein/adenylyl cyclase-cAMP/PKA) as demonstrated *in vitro*[84] and *in vivo*[83]. Oleic acid can also contribute to heart health via intramyocardial triglyceride turnover[85], which is reduced in pressure-overloaded failing hearts. In this situation oleate (derivative of oleic acid) upregulated triglyceride dynamics

when compared to palmitate (derivative of palmitic acid and major saturated fatty acid of palm oil). This result underscores the importance of the intracellular lipid storage type on nuclear receptor signaling and contractility[85] in diseased hearts.

An important driver of vasorelaxation is nitric oxide, a free radical which readily reacts with fats and proteins. Nitro-fatty acids are mediators of cardiovascular signaling actions[86] as these compounds relax blood vessels, attenuate platelet activation, and reduce inflammation[87, 88].

Both oleic acid and linoleic acid are unsaturated fatty acids that after reaction with nitrite may form nitro-fatty acids. Nitro-oleic acid mediated antihypertensive signaling actions were shown in a mouse model[89]. The mechanism was attributed to the inhibition of soluble epoxide hydrolase by nitro-fatty acids, thus lowering blood pressure in an angiotensin II-induced hypertension[89]. It is however unclear how the extent of nitrite in the human diet may contribute to nitration of dietary fat, and the physiological relevance of this finding.

Role of phenolic compounds on endothelium protection

Oxidative stress and reactive oxygen species (ROS) have been implicated in endothelial damage, progression to atherosclerosis, injury in sustained myocardial infarction and ischemia reperfusion[76, 90-92]. Monocytes and macrophages are critical cells that are involved in atherosclerosis. These cells produce proinflammatory cytokines, such as IL-1β, TNF-α and C-reactive protein (CRP), which induce the expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular-cell adhesion molecule-1 (VCAM-1), and E-selectin[93]. Meanwhile, oxidative stress through ROS production promotes the expression of the adhesion molecules on the endothelium[94].

Expression of adhesion molecules attracts circulating monocytes inducing their adherence to the endothelium. OO phenolic compounds have been shown to act on endothelium protection as evidenced in *in vitro* assays with typical OO phenolic compounds and less on *in vivo* circulating metabolites. OO phenolic extract, oleuropein aglycone or homovanillic alcohol (metabolite of hydroxytyrosol) had inhibitory effects on VCAM-1, ICAM-1 and E-selectin surface expression in human umbilical vascular endothelial cells, using TNF-α as pro-inflammatory stimulus[95].

Endothelium dysfunction refers to an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioactivity in the vessel wall. In animal models with rats oral hydroxytyrosol administration was tested on NO production and platetet function[96]. Results

showed that hydroxytyrosol administration (100 mg/kg/day) increased vascular NO production by up to 34.2% ($p < 0.01$) and inhibited platelet aggregation for 50% inhibitory dose of 48.25 mg/day for hydroxytyrosol ($p<0.01$) when compared to control group (treated with isotonic saline solution). Animal dose translation to humans allowed us to conclude that the effective hydroxytyrosol doses tested would be above the expected intake through OO daily. The reported benefits would only be achievable through nutraceutical supplementation.

Endothelium repair: matrix metalloproteinases and olive oil

Matrix metalloproteinases (MMPs) play a role in endothelium repair. Macrophages resident in human and experimental atherosclerosis co-localize with and release active MMPs including the gelatinase MMP-9, which is specialized in the digestion of basement membrane collagens and elastin, and is implicated in atherogenesis, unstable coronary syndromes, and in aortic aneurysms[97]. Accumulating evidence points to the MMPs as major molecular mediators of arterial diseases[97]. Collagens, types 1 and 3, are the main proteins in arterial walls being also present in the thickened intima of atherosclerotic lesions[98, 99]. Fragments of collagens found in urine are present as a result of proteolytic activity in arterial walls and other vascular structures. Collagen type 1 or 3 fragments were up-regulated in urine in coronary artery disease (CAD) patients[100]. Increase in collagen degradation is related with an increase on collagenases circulation, such as MMP-9, as shown in patients with CAD[101].

In an *in vitro* study hydroxytyrosol (1-10 μM) reduced MMP-9 (IC_{50} = 10 μmol/L, $p< 0.05$) and COX-2 induction in activated human monocytes, with phorbol myristate acetate (PMA)[102]. These effects were mediated by inhibition of transcription factor NF-*κ*B and protein kinase C (PKC) α and PKCβ1 activation[102]. Results are in line with previous *in vitro* reports that showed inhibition of MMP-9 on endothelial cells by OO phenolics namely hydroxytyrosol in PMA induced cells[103], and oleuropein aglycone in TNF-α induced cells by acting on NF-κB[94]. No hydroxytyrosol activity on MMP-9 was found in TNF-α induced cells[94].

The discriminatory polypetides that increase in CAD includes collagen type 1 and 3 fragments with a C-terminal GxPGP motif[104]. Increase on these polypeptides would come from a protease decrease activity possibly related with chemical change of the substrate (e.g.: oxidative damage) thus inhibiting it acting at a specific site, or a decrease in circulating levels by lack of enzyme activation. MMP-2 is secreted in an inactive form (pro MMP-2) and several factors can promote

its activation such as plasmin[105] and thrombin[106]. Other mechanisms that involve proteinases or oxidative stress can also activate MMP-2[107]. Therefore antioxidants, as phenolic compounds, might have a role on MMP-2 activation and published data indicate phenolic compounds from red wine[108] and green tea[109] as acting on prevention of thrombin-induced activation of MMP-2 in vascular smooth cells.

We evaluated the impact of a 6-week OO supplementation in healthy adults on urinary proteomic biomarkers of CAD in a randomized, parallel, controlled, double-blind study[110]. This study was the first to describe the significant impact of daily OO supplementation on highly specific disease biomarkers for CAD. Analysis of urinary proteomic profiles at baseline and endpoint enabled the identification of 12 sequenced peptides that were significantly regulated toward healthy scoring. Eight of them included four collagen α-1(I) chain, one α-2 (1) chain, one α-2(V) chain, and one α-2(VI) chain fragments. Changes in circulating concentrations of collagenases may mediate these changes in the urinary fingerprint. Therefore with more data or in future intervention studies with OO it would be interesting to link urinary fragments to the proteases involved in their generation. This predictive analysis would enable looking at the peptide cleavage sites studying the MMPs up or downregulated with OO intervention.

The majority of studies of dietary intake of proposed bioactive foods asses the activities of these foods based on the major risk factors of cardiovascular disease. However marker such as lipoprotein profile, blood pressure, endothelial function, inflammation and oxidative stress have no direct link to the disease itself but are merely associated with it. There is a great need for more biomarkers that appear as a direct result of the disease itself[63, 67].

4. Proteomics biomarkers as a mechanistic approach to explain olive oil health effects

The systems biology approach (encompassing genomics, transcriptomics, proteomics and metabolomics using urine, blood or saliva) could provide a greater understanding of disease development, treatment efficacy and evaluation of the influence of food bioactive compounds[46, 111]. There is a need for biomarkers of practical value for clinical intervention, allowing disease risk prediction and more importantly early diagnosis. Accuracy, reproducibility, availability, feasibility of implementation into the clinical settings, sensitivity and specificity are additional characteristics to be fulfilled, and panels of biomarkers are gaining acceptance instead of individual molecules[112], as single biomarkers are often not available and lack the ability to adequately describe complex diseases[113]. Candidate biomarkers should be carefully validated in a wide and different cohort of samples from those used in the discovery phase as often overfitting of the biomarker model has occurred[114].

The proteome, corresponding to a set of expressed proteins, informs the current "status" of an organism, constantly changing according to endogenous and exogenous factors[113]. Proteins are widely used in different clinical tests for both diagnosis and prognosis of diseases and to follow their evolutions[98]. They can be used to measure the extent of inflammation, calcification, and the development of plaques on the arteries. Understanding what causes plaque rupture is of great importance. As previously mentioned, MMPs could have a key role in this process[115]. The discovery of proteomic biomarkers may be useful in understanding the molecular mechanisms involved in the onset and progression of other vascular diseases[116]. Plasma, serum and urine are the most commonly used biological matrices in cardiovascular research, due to their perceived clinical relevance as a source of potential biomarkers[98]. However proteomic studies have also been carried out on vascular tissues (arteries), artery layers, cells looking at proteomes and secretomes, exosomes, lipoproteins, and metabolites[98]. Although sampling the tissue may seem an obvious method there are a number of difficulties, especially where the need for a biopsy would be required[117]. Recent advances in extraction processes and LC-MS/MS analysis has allowed the quantitative analysis of tissue samples in vascular research to be carried out[118, 119].

Urine, as a sample source is now recognized as the source of choice for proteomic biomarker investigations. It has a number of advantages such as being noninvasive and can be collected by untrained personnel. Urine is produced by renal filtration of the plasma and approximately 70%

of proteins in the normal human urinary proteome are of kidney origin, whereas the remaining 30% are derived from plasma proteins[120, 121]. It has high stability due to absence of proteolytic agents and the low dynamic range of analyte concentration facilitates the detection and quantification of peptides[113, 122].

Using capillary electrophoresis coupled with mass spectrometry (CE-MS)[123] urinary biomarker classifiers for the diagnosis of diseases like chronic kidney disease[124], acute kidney injury[125], stroke[126], and coronary artery diseases[104], were already identified, allowing classification of case *versus* control groups with good accuracy[127].

Urinary peptides and protein fragments are the end products of proteolytic processes. The different pattern of urinary excretion of peptides when comparing controls and disease patients might indicate their role in the pathophysiology of disease. Therefore changes in the normal urine "fingerprint" (e.g.: presence of collagen fragments) can be used as biomarkers of disease. Besides collagens, common blood proteins (e.g., alpha-1-antitrypsin, hemoglobin, serum albumin, and fibrinogen), and uromodulin were also identified[128] in urine which provides additional proof of the suitability of this sample source for proteomic biomarker studies out with the kidney and urinary tract. Collagens are the most abundant peptides sequenced so far in the CAD biomarker (66% of all peptides)[104], with atherosclerosis associated with an increased synthesis of several extracellular matrix components, including collagen types 1 and 3, elastin, and several proteoglycans[129]. Changes in the circulating levels of collagenases may mediate these changes in peptides represented in the fingerprint, as reported in coronary atherosclerosis[100], and chronic kidney disease[128].

The progress in urinary proteomics and the use of multiple biomarker classifiers opens the possibility of establishing new tools adapted to different clinical needs[130], enabling direct monitoring of disease overcoming limitations of indirect measurements.

Proteomic *in vitro* studies on olive oil phenolic compounds

Proteomics has been applied in a number of studies of OO phenolic compounds on cardiovascular health using animal and *in vitro* studies. The *in vitro* effects of alperujo extract, an OO production waste product containing phenolic compounds present in olive fruits, were studied on platelet aggregation and changes in the platelet proteome[131]. Nine proteins were differentially regulated by the alperujo extract upon platelet aggregation underlying the anti-

464 platelet effects of the extract. However, like a number of previously mentioned *in vitro* studies,
465 the effective concentrations (40-500 mg/L) were far above the physiologically concentrations
466 achievable by dietary intake.
467 The effects of EVOOs, with low and high in phenolic content, were evaluated in the hepatic
468 proteome in Apoe$^{-/-}$ mice that spontaneously develop atherosclerosis[132]. For 10 weeks the mice
469 were fed with a high fat high cholesterol diet supplemented with 0.15% (w/w) cholesterol and
470 either 20% (w/w) low phenolic EVOO or 20% (w/w) high phenolic EVOO *versus* a control
471 group fed with 0.15% (w/w) cholesterol and 20% (w/w) palm oil. Within this work a range of
472 hepatic antioxidant enzymes differentially regulated by OO[132] were identified. The authors
473 concluded that the up-regulation of a large array of antioxidant enzymes might explain anti-
474 atherogenic mechanisms of EVOOs[132]. Again the dose level was above what could be achieved
475 through dietary intake and translation from an animal model to human has also to be considered.
476
477 **Urinary proteomics biomarkers, olive oil and cardiovascular disease**
478 Atherosclerosis is a process of chronic inflammation, characterized by the accumulation of
479 lipids, cells, and fibrous elements in medium and large arteries[98]. The extent of
480 inflammation, proteolysis, calcification, and neovascularization influences the development of
481 advanced lesions (atheroma plaques) on the arteries[98].
482 Classical risk factors in atherosclerosis (hypertension, LDL-cholesterol, C-reactive protein,
483 aging, smoking, male gender, among others) do not actually measure disease initiation or
484 progression. As such, they cannot be used directly to identify individuals who have developed
485 atherosclerosis and prevent a fatal event[98, 133]. Other, more recent markers that indicate changes
486 in vascular structure can still only be detected once cardiovascular disease has progressed to an
487 advanced stage where drug or surgical intervention is required[134].
488 The analysis of urine samples from diseased and healthy individuals has been used to establish a
489 database of naturally occurring urinary peptides, making a basis for the definition and validation
490 of biomarkers for diagnosis/prognosis/monitoring of a wide range of diseases using proteomic
491 biomarker patterns[128], such as CAD[100], emphasizing that non-invasive proteomics analysis
492 could become a valuable addition to assess cardiovascular disease alongside to other biomarkers
493 which are indicators of cardiovascular risk.

The first time that urinary proteomics was applied to assess cardiovascular health improvements of OO consumption in humans, was in a randomized, parallel, controlled, double-blind study designed to evaluate the impact of a 6 week OO supplementation in healthy adults on urinary proteomic biomarkers of CAD[110]. The impact of the supplementation with OO was also studied on urinary proteomic biomarkers of chronic kidney disease (CKD), and diabetes.

The increase or decrease in the concentration of the peptides in the biomarker determines the scoring value of each disease biomarker. The CAD proteomic biomarker developed for clinical diagnosis produces a CAD scoring system from 1 (CAD case) to -1 (healthy artery). A scoring of disease absence, presence and severity is provided, based on the concentration of a group (panel) of urinary peptides measured by CE-MS, allowing monitoring of progression and/or effect of treatment[135, 136]. In this study, self-reported healthy participants were randomly allocated to supplementation with a daily dose of OO either low or high in phenolic compounds. For 6 weeks, they consumed a daily dose of 20 mL OO (not heated or cooked) as a supplement (no specific time during the day, single intake, equivalent to 6 mg of hydroxytyrosol and derivatives for the high phenolic OO), in line with the EFSA and FDA recommendations.The impact of supplementation with OO was evaluated on urinary proteomic biomarkers of CAD with biomarkers being measured at baseline and 3 and 6 weeks. Consumption of both OOs significantly improved the proteomic CAD score at endpoint compared with baseline, moving the CAD biomarker pattern in a healthy profile direction, **Table 2**. No differences were observed for CKD or diabetes proteomic biomarkers, Table 2.

In a placebo-controlled intervention, Irbesartan (angiotensin II receptor antagonist used for the treatment of hypertension) taken at 300 mg per day over 2 years in hypertensive type 2 diabetes patients, using the CAD 238 biomarker panel, led to a 0.35 point reduction in the CAD score for the drug-controlled group[104], which saw a significant reduction in incidents of CAD in this group. In the nutritional intervention[110] the CAD score change in the intervention was significant for both OOs tested, using the same CAD 238 biomarker, leading to a similar degree of change as observed for irbersartan over a 6 week period. This evidence highlights the importance of the CAD biomarker as a tool for nutrition and health intervention studies. This type of urinary biomarker enabled the measurement of health effects induced by a change in diet that could not be detected by monitoring the conventional risk markers of CAD such as plasma triacylglycerols, oxidized LDL, and LDL cholesterol. The overall change in CAD score in a

short period of time is more likely due to OO major components, such as fatty acids. However the role of other OO minor components other than phenolic compounds should also be taken into account. Squalene, a polyunsaturated triterpene which makes up 60–75% of the unsaponifiable fraction of OO[137], reduced atherosclerotic lesion size in male mice[138] and further investigation is needed to clarify its role on cardiovascular disease.

Our results emphasize further the potential role of nutrition in the prevention or delay of cardiovascular disease and offer new perspectives on OO applications. These results are highly translatable to guidelines for nutritional recommendations. The biomarkers were originally developed to detect early signs of diseases in clinical setting and to inform clinician as to the effectiveness of treatment. However, the technology also provides a sensitive tool for the assessment of potential bioactive foods in cardiovascular health, chronic kidney disease and diabetes, with a range of additional tests under development. Further testing of reportedly bioactive foods can now be carried out which will allow better nutritional health advice to be advanced and could also lead to better food labeling so that the public can make informed choices on their food purchases.

5. Exploring olive oil health benefits: perspectives

Although strong evidence from heritability is related with cardiovascular disease many forms of heart disease are not genome associated[139]. The epigenome is a possible link between genetics and environment[139] which includes impact of food components/diet. Omics techniques (genomics, transcriptomics, proteomics, epigenomics, metabolomics) have the potential, when integrated, to paint a comprehensive picture of the contribution of diet toward the modulation of disease risk[141]. Some trials have shown the impact of OO on down-regulation of atherosclerosis-related genes[140, 141]. The effect of Mediterranean Diet was studied on urinary metabolome[142] and related to compounds of the metabolism of carbohydrates, creatine, creatinine, amino acids, lipids and microbial cometabolites.

Phenolic compounds can interact with cellular signaling cascades regulating the activity of transcription factors with impact on gene expression. For instance, phenolic compounds have shown to affect the expression of microRNAs (miRNA)[143]. miRNAs are small, noncoding RNAs implicated in the regulation of gene expression that control both physiological and pathological processes, influenced by external factors as diet components[144]. Most of the studies reported in this field are *in vitro* and more *in vivo* studies are needed to clarify miRNA targets of dietary phenolic compounds[144].

Interactions between genes and the bioactive components present in OO studied by nutrigenomics may help to explain its health benefits[145]. In this sense, besides their antioxidant and anti-inflammatory capacities, OO phenolic compounds are able to modify gene expression coding in a protective mode for proteins participating in the cellular mechanisms involved in oxidative stress resistance, inflammation or lipid metabolism amongst others[146].

Glycation, a non-enzymatic reaction between reducing sugars and proteins, is a proteome wide phenomenon, mainly observed in diabetes due to hyperglycemia[147], but also relevant to end organ damage, disease pathogenesis and aging[148] and OO phenolic compounds have been reported as potent inhibitors of the formation of advanced glycation end products[149]. Our human intervention trial with OO low or high in phenolics did not find a significant impact on plasma fructosamine levels[110]. A key factor may be the duration of the study (6 weeks) not being sufficient to detect changes in protein modifications such as glycation, and may also be partly related to the quantity and quality of phenolic compounds, which exert differential antioxidant and antiglycative activities depending on structure[4, 150]. Further studies should proceed in

order to clarify anti-glycation properties of OO phenolic compounds, given that glycation is a key driver for tissue damage and is present in all non-communicable disease scenarios.

6. Conclusion

Results outlined in this review provide evidence of health benefits related with OO intake. The reported studies may allow the implementation of primary prevention programs of cardiovascular disease, based on nutritional interventions, useful in non-regular OO consumers groups like the Northern European populations. Interventions in broad populations with highly specific disease biomarkers, as urinary proteomic biomarkers, will offer higher translational value, especially toward development and implementation of new nutritional recommendations. Human intervention trials focusing on new outcomes related with proteomics and nutrigenomics are needed to better clarify pathways/mechanisms by which oleic acid, phenolic compounds or even other OO components act on cardiovascular disease risk factors and affect the proteome.

7. Financial Support

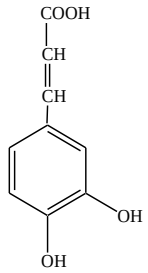
QREN project Azeite+ Global nº 12228 and Ordem dos Farmacêuticos (Lisbon, Portugal).

8. Conflict of Interest

Conflict of interest: Thomas Koeck is employed at Mosaiques Diagnostics, the company that developed the urinary proteomics for CE-MS technology for clinical application. No other authors declare a conflict of interest.

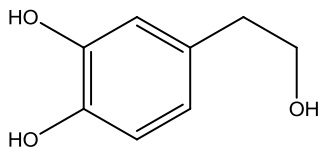
Table 1 – Main classes of phenolic compounds in virgin olive oil

Phenolic acids

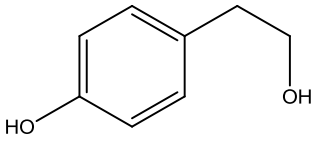


Caffeic acid

Phenolic alcohols

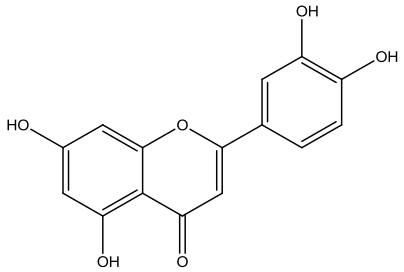


Hydroxytyrosol



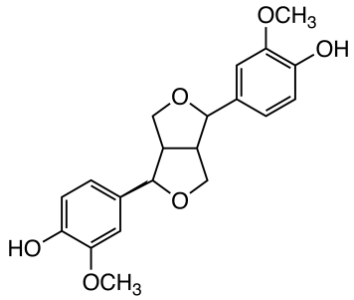
Tyrosol

Flavonoids



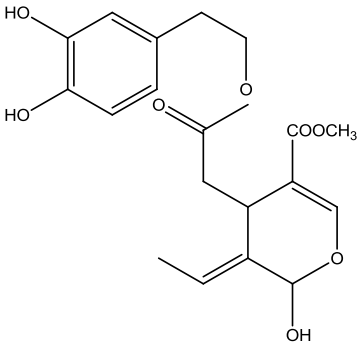
Luteolin

Lignans

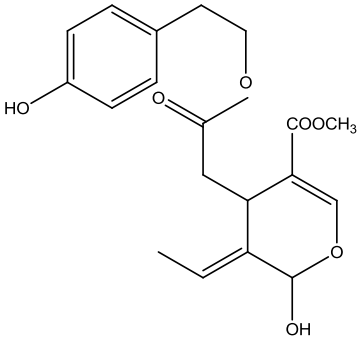


(+) - Pinoresinol

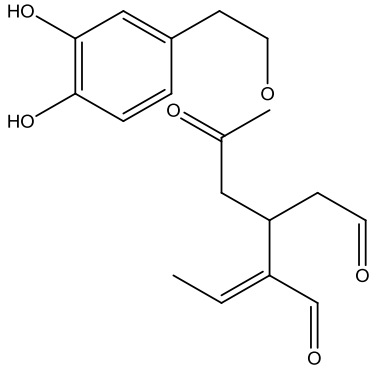
Secoiridoids



Oleuropein aglycone (3,4-DHPEA-EA)



Ligstroside aglycone (*p*-HPEA-EA)



Dialdehydic form of deacetoxy oleuropein (3,4-DHPEA-EDA)

Table 2 Changes in scores of CAD, CKD and diabetes proteomic biomarkers at baseline, middle (3-weeks) and end of intervention (6 weeks)[1]

		Low phenolic olive oil (n = 34)	High phenolic olive oil (*n* = 28)
		Score	**Score**
CAD proteomic biomarker	baseline	-0.5 ± 0.2	-0.6 ± 0.4
	3 weeks	-0.7 ± 0.3	-0.7 ± 0.3
	6 weeks	-0.8 ± 0.3**	-0.8 ± 0.3*
CKD proteomic biomarker	baseline	-0.4 ± 0.2	-0.4 ± 0.3
	3 weeks	-0.4 ± 0.2	-0.4 ± 0.3
	6 weeks	-0.4 ± 0.2	-0.4 ± 0.2
Diabetes proteomic biomarker	baseline	1.3 ± 0.3	1.3 ± 0.3
	3 weeks	1.3 ± 0.4	1.3 ± 0.3
	6 weeks	1.4 ± 0.4	1.2 ± 0.3

[1]Values are means ± SDs; 95% CIs in parentheses. A repeated-measures ANOVA test was used with statistical significance at $p < 0.05$. ***Compared with corresponding baseline value: *$p < 0.005$, **$p < 0.001$. There were no significant differences in changes between groups. CAD, coronary artery disease; CKD, chronic kidney disease (adapted from Silva *et al.*[(104)]).

REFERENCES

1. Obied HK, Prenzler PD, Ryan D, Servili M, Taticchi A, Esposto S, et al. Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from Olea europaea L. Natural product reports. 2008;25(6):1167-79.
2. European Comission, Official Journal of the European Union, 27.1.2011, Comission Regulation (EC) No 61/2001 of 24 January 2011
3. European Comission, Official Journal of the European Union, 5.7.2008, Comission Regulation (EC) No 640/2008 of 4 July 2008.
4. Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S, La Guardia M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutrition research reviews. 2005;18(1):98-112.
5. Covas MI. Olive oil and the cardiovascular system. Pharmacological research : the official journal of the Italian Pharmacological Society. 2007;55(3):175-86.
6. Lopez-Miranda J, Perez-Jimenez F, Ros E, De Caterina R, Badimon L, Covas MI, et al. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2010;20(4):284-94.
7. Urpi-Sarda M, Casas R, Chiva-Blanch G, Romero-Mamani ES, Valderas-Martinez P, Arranz S, et al. Virgin olive oil and nuts as key foods of the Mediterranean diet effects on inflammatory biomakers related to atherosclerosis. Pharmacological research : the official journal of the Italian Pharmacological Society. 2012;65(6):577-83.
8. Martinez-Lapiscina EH, Clavero P, Toledo E, San Julian B, Sanchez-Tainta A, Corella D, et al. Virgin olive oil supplementation and long-term cognition: the PREDIMED-NAVARRA randomized, trial. The journal of nutrition, health & aging. 2013;17(6):544-52.
9. Kastorini CM, Milionis HJ, Goudevenos JA, Panagiotakos DB. Mediterranean diet and coronary heart disease: is obesity a link? - A systematic review. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2010;20(7):536-51.
10. Razquin C, Martinez JA, Martinez-Gonzalez MA, Mitjavila MT, Estruch R, Marti A. A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. European journal of clinical nutrition. 2009;63(12):1387-93.
11. Schwingshackl L, Hoffmann G. Mediterranean dietary pattern, inflammation and endothelial function: a systematic review and meta-analysis of intervention trials. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2014;24(9):929-39.
12. Schwingshackl L, Hoffmann G. Monounsaturated fatty acids, olive oil and health status: a systematic review and meta-analysis of cohort studies. Lipids in health and disease. 2014;13:154.
13. Harwood J. AR. Handbook of olive oil analysis and properties 2000.
14. Servili M, Montedoro, G. . Contribution of phenolic compounds to virgin olive oil quality. European Journal of Lipid Science and Technology. 2002;104:602-13.
15. Perez-Jimenez F, Ruano J, Perez-Martinez P, Lopez-Segura F, Lopez-Miranda J. The influence of olive oil on human health: not a question of fat alone. Molecular nutrition & food research. 2007;51(10):1199-208.

16. Di Maio I, Esposto, S., Taticchi, A., Selvaggini, R., Veneziani, G., Urbani, S. , Servili, M. . HPLC–ESI-MS investigation of tyrosol and hydroxytyrosol oxidation products in virgin olive oil. Food Chemistry. 2011;125:21-8.
17. Brenes M, Garcia A, Garcia P, Garrido A. Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. Journal of agricultural and food chemistry. 2001;49(11):5609-14.
18. Hrncirik K. F, S. . Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. European Journal of Lipid Science and Technology. 2004;106:540-9.
19. Karkoula E, Skantzari A, Melliou E, Magiatis P. Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative (1)H NMR. Establishment of a new index for the characterization of extra virgin olive oils. Journal of agricultural and food chemistry. 2012;60(47):11696-703.
20. Corona G, Tzounis X, Assunta Dessi M, Deiana M, Debnam ES, Visioli F, et al. The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. Free radical research. 2006;40(6):647-58.
21. Pinto J, Paiva-Martins F, Corona G, Debnam ES, Jose Oruna-Concha M, Vauzour D, et al. Absorption and metabolism of olive oil secoiridoids in the small intestine. The British journal of nutrition. 2011;105(11):1607-18.
22. Aranzazu Soler MPR, Alba Macià, Shikha Saha, Caroline S.M. Furniss, Paul A. Kroon, Maria J. Motilva. Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. Food Chemistry. 2010;119:703-14.
23. Vissers MN, Zock PL, Roodenburg AJ, Leenen R, Katan MB. Olive oil phenols are absorbed in humans. The Journal of nutrition. 2002;132(3):409-17.
24. Garcia-Villalba R, Carrasco-Pancorbo A, Nevedomskaya E, Mayboroda OA, Deelder AM, Segura-Carretero A, et al. Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil: understanding the metabolism of polyphenols. Analytical and bioanalytical chemistry. 2010;398(1):463-75.
25. Mateos R, Goya L, Bravo L. Metabolism of the olive oil phenols hydroxytyrosol, tyrosol, and hydroxytyrosyl acetate by human hepatoma HepG2 cells. Journal of agricultural and food chemistry. 2005;53(26):9897-905.
26. Miro-Casas E, Covas MI, Farre M, Fito M, Ortuno J, Weinbrenner T, et al. Hydroxytyrosol disposition in humans. Clinical chemistry. 2003;49(6 Pt 1):945-52.
27. Miro Casas E, Farre Albadalejo M, Covas Planells MI, Fito Colomer M, Lamuela Raventos RM, de la Torre Fornell R. Tyrosol bioavailability in humans after ingestion of virgin olive oil. Clinical chemistry. 2001;47(2):341-3.
28. Garcia-Villalba R, Larrosa M, Possemiers S, Tomas-Barberan FA, Espin JC. Bioavailability of phenolics from an oleuropein-rich olive (Olea europaea) leaf extract and its acute effect on plasma antioxidant status: comparison between pre- and postmenopausal women. European journal of nutrition. 2014;53(4):1015-27.
29. Suarez M, Valls RM, Romero MP, Macia A, Fernandez S, Giralt M, et al. Bioavailability of phenols from a phenol-enriched olive oil. The British journal of nutrition. 2011;106(11):1691-701.
30. Preedy V.R. WRR. Olives and olive oil in health and disease prevention. 1st ed2010.

31. Tuck KL, Freeman MP, Hayball PJ, Stretch GL, Stupans I. The in vivo fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats. The Journal of nutrition. 2001;131(7):1993-6.
32. Gonzalez-Santiago M, Fonolla J, Lopez-Huertas E. Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. Pharmacological research : the official journal of the Italian Pharmacological Society. 2010;61(4):364-70.
33. Visioli F, Galli C, Grande S, Colonnelli K, Patelli C, Galli G, et al. Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. The Journal of nutrition. 2003;133(8):2612-5.
34. Mosele JI, Martin-Pelaez S, Macia A, Farras M, Valls RM, Catalan U, et al. Faecal microbial metabolism of olive oil phenolic compounds: in vitro and in vivo approaches. Molecular nutrition & food research. 2014;58(9):1809-19.
35. Khymenets O, Fito M, Tourino S, Munoz-Aguayo D, Pujadas M, Torres JL, et al. Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion. Drug metabolism and disposition: the biological fate of chemicals. 2010;38(9):1417-21.
36. Tuck KL, Hayball PJ, Stupans I. Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. Journal of agricultural and food chemistry. 2002;50(8):2404-9.
37. Kotronoulas A, Pizarro N, Serra A, Robledo P, Joglar J, Rubio L, et al. Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. Pharmacological research : the official journal of the Italian Pharmacological Society. 2013;77:47-56.
38. Laura Rubió AS, Alba Macià, Carme Piñol, Maria-Paz Romero, Maria-José Motilva. In vivo distribution and deconjugation of hydroxytyrosol phase II metabolites in red blood cells: A potential new target for hydroxytyrosol. Journal of functional foods. 2014;10:139-43.
39. Hamden K, Allouche N, Damak M, Elfeki A. Hypoglycemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste in vitro and in rats. Chemico-biological interactions. 2009;180(3):421-32.
40. Sanchez de Medina V, Priego-Capote F, Luque de Castro MD. Characterization of refined edible oils enriched with phenolic extracts from olive leaves and pomace. Journal of agricultural and food chemistry. 2012;60(23):5866-73.
41. Suarez M, Romero MP, Motilva MJ. Development of a phenol-enriched olive oil with phenolic compounds from olive cake. Journal of agricultural and food chemistry. 2010;58(19):10396-403.
42. Pashkow FJ. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? International journal of inflammation. 2011;2011:514623.
43. Vlantis K, Pasparakis M. Role of TNF in pathologies induced by nuclear factor kappaB deficiency. Current directions in autoimmunity. 2010;11:80-93.
44. Beauchamp GK, Keast RS, Morel D, Lin J, Pika J, Han Q, et al. Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. Nature. 2005;437(7055):45-6.
45. Beauchamp GK, Keast RS, Morel D, Lin J, Pika J, Han Q, et al. Ibuprofen-like activity in extra-virgin olive oil. Nature. 2005;437(7055):45-6.
46. Tulp M, Bruhn JG, Bohlin L. Food for thought. Drug discovery today. 2006;11(23-24):1115-21.

47. O'Connor Á. An overview of the role of diet in the treatment of rheumatoid arthritis. Nutrition Bulletin. 2014;39(1):74-88.
48. Waterman E, Lockwood B. Active components and clinical applications of olive oil. Altern Med Rev. 2007;12(4):331-42.
49. S. Silva BS, J. Rocha, R. Direito, A. Fernandes, D. Brites, M. Freitas, E. Fernandes, M. R. Bronze, M. E. Figueira. Protective effects of hydroxytyrosol-supplemented refined olive oil in animal models of acute inflammation and rheumatoid arthritis The Journal of nutritional biochemistry. 2015.
50. European Comission, Comission Regulation (EC) No 432/2012 of 16 May 2012.
51. Sanchez-Fidalgo S, Sanchez de Ibarguen L, Cardeno A, Alarcon de la Lastra C. Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model. European journal of nutrition. 2012;51(4):497-506.
52. Rosillo MA, Alcaraz MJ, Sanchez-Hidalgo M, Fernandez-Bolanos JG, Alarcon-de-la-Lastra C, Ferrandiz ML. Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis. The Journal of nutritional biochemistry. 2014;25(12):1275-81.
53. Impellizzeri D, Esposito E, Mazzon E, Paterniti I, Di Paola R, Morittu VM, et al. Oleuropein aglycone, an olive oil compound, ameliorates development of arthritis caused by injection of collagen type II in mice. The Journal of pharmacology and experimental therapeutics. 2011;339(3):859-69.
54. Martinez-Dominguez E, de la Puerta R, Ruiz-Gutierrez V. Protective effects upon experimental inflammation models of a polyphenol-supplemented virgin olive oil diet. Inflammation research : official journal of the European Histamine Research Society [et al]. 2001;50(2):102-6.
55. Bignotto L, Rocha J, Sepodes B, Eduardo-Figueira M, Pinto R, Chaud M, et al. Anti-inflammatory effect of lycopene on carrageenan-induced paw oedema and hepatic ischaemia-reperfusion in the rat. The British journal of nutrition. 2009;102(1):126-33.
56. Gong D, Geng C, Jiang L, Cao J, Yoshimura H, Zhong L. Effects of hydroxytyrosol-20 on carrageenan-induced acute inflammation and hyperalgesia in rats. Phytotherapy research : PTR. 2009;23(5):646-50.
57. Gimeno E, de la Torre-Carbot K, Lamuela-Raventos RM, Castellote AI, Fito M, de la Torre R, et al. Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. The British journal of nutrition. 2007;98(6):1243-50.
58. Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, et al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Annals of internal medicine. 2006;145(5):333-41.
59. Covas MI, de la Torre K, Farre-Albaladejo M, Kaikkonen J, Fito M, Lopez-Sabater C, et al. Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. Free radical biology & medicine. 2006;40(4):608-16.
60. Fito M, Cladellas M, de la Torre R, Marti J, Alcantara M, Pujadas-Bastardes M, et al. Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomized, crossover, controlled, clinical trial. Atherosclerosis. 2005;181(1):149-58.
61. Weinbrenner T, Fito M, de la Torre R, Saez GT, Rijken P, Tormos C, et al. Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. The Journal of nutrition. 2004;134(9):2314-21.

62. Marrugat J, Covas MI, Fito M, Schroder H, Miro-Casas E, Gimeno E, et al. Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation--a randomized controlled trial. European journal of nutrition. 2004;43(3):140-7.
63. Ruiz-Canela M, Martinez-Gonzalez MA. Olive oil in the primary prevention of cardiovascular disease. Maturitas. 2011;68(3):245-50.
64. Katan MB, Zock PL, Mensink RP. Effects of fats and fatty acids on blood lipids in humans: an overview. The American journal of clinical nutrition. 1994;60(6 Suppl):1017S-22S.
65. Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA Study. Journal of the American College of Cardiology. 2004;44(1):152-8.
66. Huang CL, Sumpio BE. Olive oil, the mediterranean diet, and cardiovascular health. Journal of the American College of Surgeons. 2008;207(3):407-16.
67. Covas MI, Konstantinidou V, Fito M. Olive oil and cardiovascular health. Journal of cardiovascular pharmacology. 2009;54(6):477-82.
68. Krauss RM, Dreon DM. Low-density-lipoprotein subclasses and response to a low-fat diet in healthy men. The American journal of clinical nutrition. 1995;62(2):478S-87S.
69. Bos G, Poortvliet MC, Scheffer PG, Dekker JM, Ocke MC, Nijpels G, et al. Dietary polyunsaturated fat intake is associated with low-density lipoprotein size, but not with susceptibility to oxidation in subjects with impaired glucose metabolism and type II diabetes: the Hoorn study. European journal of clinical nutrition. 2007;61(2):205-11.
70. Chait A, Brazg RL, Tribble DL, Krauss RM. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. The American journal of medicine. 1993;94(4):350-6.
71. Aguilera CM, Mesa MD, Ramirez-Tortosa MC, Nestares MT, Ros E, Gil A. Sunflower oil does not protect against LDL oxidation as virgin olive oil does in patients with peripheral vascular disease. Clinical nutrition. 2004;23(4):673-81.
72. Kratz M, Cullen P, Kannenberg F, Kassner A, Fobker M, Abuja PM, et al. Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. European journal of clinical nutrition. 2002;56(1):72-81.
73. FDA. FDA Allows Qualified Health Claim to Decrease Risk of Coronary Heart Disease 2004. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2004/ucm108368.htm].
74. Fuentes F, Lopez-Miranda J, Perez-Martinez P, Jimenez Y, Marin C, Gomez P, et al. Chronic effects of a high-fat diet enriched with virgin olive oil and a low-fat diet enriched with alpha-linolenic acid on postprandial endothelial function in healthy men. The British journal of nutrition. 2008;100(1):159-65.
75. Capurso C, Massaro M, Scoditti E, Vendemiale G, Capurso A. Vascular effects of the Mediterranean diet Part I: Anti-hypertensive and anti-thrombotic effects. Vascular pharmacology. 2014;63(3):118-26.
76. Khurana S, Venkataraman K, Hollingsworth A, Piche M, Tai TC. Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients. 2013;5(10):3779-827.
77. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), “anti-inflammatory properties” (ID 1882), “contributes to the upper respiratory tract health” (ID 3468), “can help to maintain a normal function of gastrointestinal

tract" (3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/20061. EFSA Journal. 2011;9 (4)(2033).
78. Scientific Opinion on the substantiation of health claims related to olive oil and maintenance of normal blood LDL-cholesterol concentrations (ID 1316, 1332), maintenance of normal (fasting) blood concentrations of triglycerides (ID 1316, 1332), maintenance of normal blood HDL-cholesterol concentrations (ID 1316, 1332) and maintenance of normal blood glucose concentrations (ID 4244) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal. 2011 9 (4)(2044).
79. Vissers MN, Zock PL, Katan MB. Bioavailability and antioxidant effects of olive oil phenols in humans: a review. European journal of clinical nutrition. 2004;58(6):955-65.
80. Rietjens SJ, Bast A, Haenen GR. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. Journal of agricultural and food chemistry. 2007;55(18):7609-14.
81. Mastralexi A, Nenadis N, Tsimidou MZ. Addressing analytical requirements to support health claims on "olive oil polyphenols" (EC Regulation 432/2012). Journal of agricultural and food chemistry. 2014;62(12):2459-61.
82. Romero C, Brenes M. Analysis of total contents of hydroxytyrosol and tyrosol in olive oils. Journal of agricultural and food chemistry. 2012;60(36):9017-22.
83. Teres S, Barcelo-Coblijn G, Benet M, Alvarez R, Bressani R, Halver JE, et al. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(37):13811-6.
84. Yang Q, Alemany R, Casas J, Kitajka K, Lanier SM, Escriba PV. Influence of the membrane lipid structure on signal processing via G protein-coupled receptors. Molecular pharmacology. 2005;68(1):210-7.
85. Lahey R, Wang X, Carley AN, Lewandowski ED. Dietary fat supply to failing hearts determines dynamic lipid signaling for nuclear receptor activation and oxidation of stored triglyceride. Circulation. 2014;130(20):1790-9.
86. Rudolph V, Rudolph TK, Schopfer FJ, Bonacci G, Woodcock SR, Cole MP, et al. Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. Cardiovascular research. 2010;85(1):155-66.
87. Coles B, Bloodsworth A, Clark SR, Lewis MJ, Cross AR, Freeman BA, et al. Nitrolinoleate inhibits superoxide generation, degranulation, and integrin expression by human neutrophils: novel antiinflammatory properties of nitric oxide-derived reactive species in vascular cells. Circulation research. 2002;91(5):375-81.
88. Coles B, Bloodsworth A, Eiserich JP, Coffey MJ, McLoughlin RM, Giddings JC, et al. Nitrolinoleate inhibits platelet activation by attenuating calcium mobilization and inducing phosphorylation of vasodilator-stimulated phosphoprotein through elevation of cAMP. The Journal of biological chemistry. 2002;277(8):5832-40.
89. Charles RL, Rudyk O, Prysyazhna O, Kamynina A, Yang J, Morisseau C, et al. Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(22):8167-72.
90. Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. Journal of hypertension. 2000;18(6):655-73.
91. Sugamura K, Keaney JF, Jr. Reactive oxygen species in cardiovascular disease. Free radical biology & medicine. 2011;51(5):978-92.

92. Raedschelders K, Ansley DM, Chen DD. The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacology & therapeutics. 2012;133(2):230-55.
93. Ross R. Atherosclerosis--an inflammatory disease. The New England journal of medicine. 1999;340(2):115-26.
94. Dell'Agli M, Fagnani R, Galli GV, Maschi O, Gilardi F, Bellosta S, et al. Olive oil phenols modulate the expression of metalloproteinase 9 in THP-1 cells by acting on nuclear factor-kappaB signaling. Journal of agricultural and food chemistry. 2010;58(4):2246-52.
95. Dell'Agli M, Fagnani R, Mitro N, Scurati S, Masciadri M, Mussoni L, et al. Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. Journal of agricultural and food chemistry. 2006;54(9):3259-64.
96. Gonzalez-Correa JA, Navas MD, Munoz-Marin J, Trujillo M, Fernandez-Bolanos J, de la Cruz JP. Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. Journal of agricultural and food chemistry. 2008;56(17):7872-6.
97. Dollery CM, Libby P. Atherosclerosis and proteinase activation. Cardiovascular research. 2006;69(3):625-35.
98. Barderas MG, Vivanco F, Alvarez-Llamas G. Vascular proteomics. Methods in molecular biology. 2013;1000:1-20.
99. von Zur Muhlen C, Schiffer E, Zuerbig P, Kellmann M, Brasse M, Meert N, et al. Evaluation of urine proteome pattern analysis for its potential to reflect coronary artery atherosclerosis in symptomatic patients. Journal of proteome research. 2009;8(1):335-45.
100. Zimmerli LU, Schiffer E, Zurbig P, Good DM, Kellmann M, Mouls L, et al. Urinary proteomic biomarkers in coronary artery disease. Molecular & cellular proteomics : MCP. 2008;7(2):290-8.
101. Kalela A, Koivu TA, Sisto T, Kanervisto J, Hoyhtya M, Sillanaukee P, et al. Serum matrix metalloproteinase-9 concentration in angiographically assessed coronary artery disease. Scandinavian journal of clinical and laboratory investigation. 2002;62(5):337-42.
102. Scoditti E, Nestola A, Massaro M, Calabriso N, Storelli C, De Caterina R, et al. Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKCalpha and PKCbeta1 inhibition. Atherosclerosis. 2014;232(1):17-24.
103. Scoditti E, Calabriso N, Massaro M, Pellegrino M, Storelli C, Martines G, et al. Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a potentially protective mechanism in atherosclerotic vascular disease and cancer. Archives of biochemistry and biophysics. 2012;527(2):81-9.
104. Delles C, Schiffer E, von Zur Muhlen C, Peter K, Rossing P, Parving HH, et al. Urinary proteomic diagnosis of coronary artery disease: identification and clinical validation in 623 individuals. Journal of hypertension. 2010;28(11):2316-22.
105. Monea S, Lehti K, Keski-Oja J, Mignatti P. Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism. Journal of cellular physiology. 2002;192(2):160-70.
106. Lafleur MA, Hollenberg MD, Atkinson SJ, Knauper V, Murphy G, Edwards DR. Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species. The Biochemical journal. 2001;357(Pt 1):107-15.

107. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. The Journal of clinical investigation. 1996;98(11):2572-9.
108. Oak MH, El Bedoui J, Anglard P, Schini-Kerth VB. Red wine polyphenolic compounds strongly inhibit pro-matrix metalloproteinase-2 expression and its activation in response to thrombin via direct inhibition of membrane type 1-matrix metalloproteinase in vascular smooth muscle cells. Circulation. 2004;110(13):1861-7.
109. El Bedoui J, Oak MH, Anglard P, Schini-Kerth VB. Catechins prevent vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression. Cardiovascular research. 2005;67(2):317-25.
110. Silva S, Bronze MR, Figueira ME, Siwy J, Mischak H, Combet E, et al. Impact of a 6-wk olive oil supplementation in healthy adults on urinary proteomic biomarkers of coronary artery disease, chronic kidney disease, and diabetes (types 1 and 2): a randomized, parallel, controlled, double-blind study. The American journal of clinical nutrition. 2015;101(1):44-54.
111. Wang M, Lamers RJ, Korthout HA, van Nesselrooij JH, Witkamp RF, van der Heijden R, et al. Metabolomics in the context of systems biology: bridging traditional Chinese medicine and molecular pharmacology. Phytotherapy research : PTR. 2005;19(3):173-82.
112. Finley Austin MJ, Babiss L. Commentary: where and how could biomarkers be used in 2016? The AAPS journal. 2006;8(1):E185-9.
113. Schanstra JP, Mischak H. Proteomic urinary biomarker approach in renal disease: from discovery to implementation. Pediatric nephrology. 2014.
114. Mischak H, Allmaier G, Apweiler R, Attwood T, Baumann M, Benigni A, et al. Recommendations for biomarker identification and qualification in clinical proteomics. Science translational medicine. 2010;2(46):46ps2.
115. Stegemann C, Didangelos A, Barallobre-Barreiro J, Langley SR, Mandal K, Jahangiri M, et al. Proteomic identification of matrix metalloproteinase substrates in the human vasculature. Circ-Cardiovasc Gene. 2013;6(Article):106-17.
116. Mischak H, Rossing P. Proteomic biomarkers in diabetic nephropathy--reality or future promise? Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010;25(9):2843-5.
117. Lescuyer P, Hochstrasser D, Rabilloud T. How shall we use the proteomics toolbox for biomarker discovery? J Proteome Res. 2007;6(9):3371-6.
118. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359-62.
119. Husi H, Van Agtmael T, Mullen W, Bahlmann FH, Schanstra JP, Vlahou A, et al. Proteome-Based Systems Biology Analysis of the Diabetic Mouse Aorta Reveals Major Changes in Fatty Acid Biosynthesis as Potential Hallmark in Diabetes Mellitus-Associated Vascular Disease. Circ-Cardiovasc Gene. 2014;7(2):161-70.
120. Thongboonkerd V, McLeish KR, Arthur JM, Klein JB. Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney international. 2002;62(4):1461-9.
121. Thongboonkerd V, Malasit P. Renal and urinary proteomics: current applications and challenges. Proteomics. 2005;5(4):1033-42.

122. Mischak H, Kolch W, Aivaliotis M, Bouyssie D, Court M, Dihazi H, et al. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. Proteomics Clinical applications. 2010;4(4):464-78.
123. Albalat A, Franke J, Gonzalez J, Mischak H, Zurbig P. Urinary proteomics based on capillary electrophoresis coupled to mass spectrometry in kidney disease. Methods in molecular biology. 2013;919:203-13.
124. Good DM, Zurbig P, Argiles A, Bauer HW, Behrens G, Coon JJ, et al. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Molecular & cellular proteomics : MCP. 2010;9(11):2424-37.
125. Metzger J, Kirsch T, Schiffer E, Ulger P, Mentes E, Brand K, et al. Urinary excretion of twenty peptides forms an early and accurate diagnostic pattern of acute kidney injury. Kidney international. 2010;78(12):1252-62.
126. Dawson J, Walters M, Delles C, Mischak H, Mullen W. Urinary proteomics to support diagnosis of stroke. PloS one. 2012;7(5):e35879.
127. Mischak H, Schanstra JP. CE-MS in biomarker discovery, validation, and clinical application. Proteomics Clinical applications. 2011;5(1-2):9-23.
128. Coon JJ, Zurbig P, Dakna M, Dominiczak AF, Decramer S, Fliser D, et al. CE-MS analysis of the human urinary proteome for biomarker discovery and disease diagnostics. Proteomics Clinical applications. 2008;2(7-8):964.
129. Lee RT, Libby P. The unstable atheroma. Arteriosclerosis, thrombosis, and vascular biology. 1997;17(10):1859-67.
130. Zurbig P, Jerums G, Hovind P, Macisaac RJ, Mischak H, Nielsen SE, et al. Urinary proteomics for early diagnosis in diabetic nephropathy. Diabetes. 2012;61(12):3304-13.
131. de Roos B, Zhang X, Rodriguez Gutierrez G, Wood S, Rucklidge GJ, Reid MD, et al. Anti-platelet effects of olive oil extract: in vitro functional and proteomic studies. European journal of nutrition. 2011;50(7):553-62.
132. Arbones-Mainar JM, Ross K, Rucklidge GJ, Reid M, Duncan G, Arthur JR, et al. Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE-/- mice. Journal of proteome research. 2007;6(10):4041-54.
133. Ge Y, Wang TJ. Identifying novel biomarkers for cardiovascular disease risk prediction. Journal of internal medicine. 2012;272(5):430-9.
134. Sharma P, Cosme J, Gramolini AO. Recent advances in cardiovascular proteomics. J Proteomics. 2013;81:3-14.
135. Fliser D, Novak J, Thongboonkerd V, Argiles A, Jankowski V, Girolami MA, et al. Advances in urinary proteome analysis and biomarker discovery. Journal of the American Society of Nephrology. 2007;18(4):1057-71.
136. Julian BA, Suzuki H, Suzuki Y, Tomino Y, Spasovski G, Novak J. Sources of urinary proteins and their analysis by urinary proteomics for the detection of biomarkers of disease. Proteom Clin Appl. 2009;3(9):1029-43.
137. Perona JS, Cabello-Moruno R, Ruiz-Gutierrez V. The role of virgin olive oil components in the modulation of endothelial function. The Journal of nutritional biochemistry. 2006;17(7):429-45.
138. Guillen N, Acin S, Navarro MA, Perona JS, Arbones-Mainar JM, Arnal C, et al. Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice. Atherosclerosis. 2008;197(1):72-83.

139. Monte E, Vondriska TM. Epigenomes: the missing heritability in human cardiovascular disease? Proteomics Clinical applications. 2014;8(7-8):480-7.
140. Konstantinidou V, Covas MI, Munoz-Aguayo D, Khymenets O, de la Torre R, Saez G, et al. In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2010;24(7):2546-57.
141. Camargo A, Ruano J, Fernandez JM, Parnell LD, Jimenez A, Santos-Gonzalez M, et al. Gene expression changes in mononuclear cells in patients with metabolic syndrome after acute intake of phenol-rich virgin olive oil. BMC genomics. 2010;11:253.
142. Vazquez-Fresno R, Llorach R, Urpi-Sarda M, Lupianez-Barbero A, Estruch R, Corella D, et al. Metabolomic Pattern Analysis after Mediterranean Diet Intervention in a Nondiabetic Population: A 1- and 3-Year Follow-up in the PREDIMED Study. Journal of proteome research. 2015;14(1):531-40.
143. Noratto GD, Angel-Morales G, Talcott ST, Mertens-Talcott SU. Polyphenolics from acai (Euterpe oleracea Mart.) and red muscadine grape (Vitis rotundifolia) protect human umbilical vascular Endothelial cells (HUVEC) from glucose- and lipopolysaccharide (LPS)-induced inflammation and target microRNA-126. Journal of agricultural and food chemistry. 2011;59(14):7999-8012.
144. Milenkovic D, Jude B, Morand C. miRNA as molecular target of polyphenols underlying their biological effects. Free radical biology & medicine. 2013;64:40-51.
145. Garcia-Gonzalez DL, Aparicio R. Research in olive oil: challenges for the near future. Journal of agricultural and food chemistry. 2010;58(24):12569-77.
146. Martin-Pelaez S, Covas MI, Fito M, Kusar A, Pravst I. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Molecular nutrition & food research. 2013;57(5):760-71.
147. Dunn MJ. Proteomics clinical applications reviews 2013. Proteomics Clinical applications. 2013;7(1-2):4-7.
148. Levi B, Werman MJ. Long-term fructose consumption accelerates glycation and several age-related variables in male rats. J Nutr. 1998;128(9):1442-9.
149. Kontogianni VG, Charisiadis P, Margianni E, Lamari FN, Gerothanassis IP, Tzakos AG. Olive leaf extracts are a natural source of advanced glycation end product inhibitors. Journal of medicinal food. 2013;16(9):817-22.
150. Vlassopoulos A, Lean ME, Combet E. Protein-phenolic interactions and inhibition of glycation - combining a systematic review and experimental models for enhanced physiological relevance. Food & function. 2014;5(10):2646-55.

Figure 1 – Chronic inflammation model and impact on rats paw edema (ANOVA, * $p < 0.001$ *vs.* postive control – Rheumatoid Arthritis, + $p < 0.01$ *vs.* Refined Olive Oil; OHTYR = hydroxytyrosol) (adapted from Silva *et al.*[49])